SOLUBILITY CHANGES OF PROTEINS IN SEA URCHIN EGGS UPON FERTILIZATION

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The electrophoretic analysis of the water-extractable proteins of the eggs of the sea urchin *Paracentrotus lividus* has given evidence of a process of rearrangement of the protein pattern of the egg taking place in the very early stages following fertilization (Monroy, 1950). In this process mainly solubility changes are involved (Monroy, 1950; Lindvall and Carsjö, 1950). Changes in solubility of the egg proteins as a result of fertilization were first described by Mirsky (1936). In the eggs of *Arbacia punctulata* and *Stronglylocentrotus purpuratus* Mirsky found that a protein fraction, amounting to about 12 per cent of the total proteins of the egg, becomes insoluble in 1 M KCl during the first 10 minutes following fertilization.

The results presented in this paper deal with some solubility changes observed in a protein fraction of the eggs of *Arbacia lixula* as a result of fertilization. Some observations on the eggs of *Arbacia punctulata* will also be referred to. However, the lability of the protein system involved in the change and the possibility of artifacts interfering with the results have made it difficult so far to analyze the process in greater detail.

EXPERIMENTAL

The eggs of *Arbacia lixula* were collected during the spring of 1949 at the Zoological Station, Naples. The gonads were removed from the animals and shaken in sea water to release the eggs. The eggs were then filtered through cheese-cloth to remove tissue debris. After a few washings with sea water, the eggs were centrifuged down, frozen in solid CO_2-acetone mixture, and dried in the high vacuum. Fertilized eggs were collected at different time intervals after fertilization. Fertilization, stage of development at the moment of sampling, and percentage of developing eggs were always checked under the microscope. All the extraction procedures were carried out in the cold room at about +4° C. The dry eggs were first taken up in a small amount of distilled water and homogenized for a couple of minutes in a glass homogenizer with a slowly rotating motor-driven glass plunger. The extraction was then completed with the desired amount of distilled water, stirring the suspension at intervals of a few minutes, for

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about 20 to 30 minutes. During the extraction the tubes were kept in ice. The extracts were centrifuged at 7000 R.P.M. in the cold, the water extract was carefully pipetted out and collected, and the sediment rapidly washed with distilled water and recentrifuged. The sediment was finally extracted with 1 M KCl. For the electrophoretic analysis Longsworth's technique was used. The extracts were dialyzed against three changes of 0.1 N $NaHCO_3$, pH 8.6, for a period of 48 hours. The photographs of the patterns were enlarged on millimeter paper and used for measurements of the relative areas and mobilities.

N was estimated by direct Nesslerization, reading the color in the spectrophotometer.

TABLE I

Relative Concentrations of Protein Components in Water Extracts of Eggs of Arbacia lixula

Concentrations in per cent of the total proteins.

Preparations	Components				
	1 $u = -9.2$	2 $u = -6.8$	3 $u = -6.2$	4 $u = -5.5$	5 $u = -4.8$
44,1 unfertilized....	17.3	7.0	8.5	16.2	51.0
44,3 45 min. after fertilization......	13.6	9.2	11.0	18.0	49.0
42,1 unfertilized....	16.3	7.5	11.5	14.8	61.0
42,2 5 min. after fertilization......	13.0	13.7	13.3	17.9	49.0
42,3 45 min. after fertilization......	17.8	9.4	10.0	16.0	52.0

Electrophoresis in 0.1 N $NaHCO_3$, pH 8.6, ionic strength 0.1, potential gradient 4.6 volts/cm.

RESULTS

In contrast with our earlier results on *Paracentrotus lividus*, the electrophoretic patterns of the water extracts of unfertilized and fertilized eggs of *Arbacia lixula* are essentially identical (Table I and Fig. 1). The small differences in the relative areas of some components before and after fertilization need to be studied further with larger quantities of material. The pattern given by the 1 M KCl soluble fraction (after removal of the water-soluble fractions) shows a decrease in the relative area of component *b*, without any change in mobility (Fig. 2 and Table II).

In two experiments, at the end of the electrophoretic run, the group of the components *a* and *b* and the main component *c* of the KCl fraction were withdrawn separately from the two channels and analyzed in the ultraviolet. As shown in Fig. 3, the group of components *a* and *b* gives an absorption spectrum with a maximum at 258 mμ, while the main component gives a typical protein spectrum.

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In Table III the data are summarized for the N content of the water and KCl extracts (undialyzed) and for the residue insoluble in 1 M KCl of un-

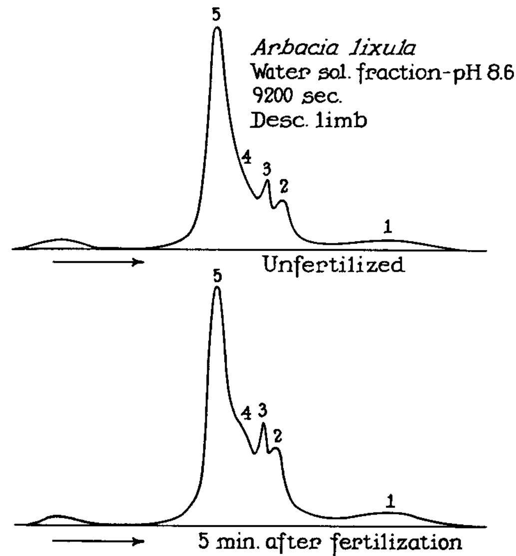

FIG. 1. Electrophoretic diagrams of the water-soluble fraction of unfertilized and fertilized eggs of *Arbacia lixula*.

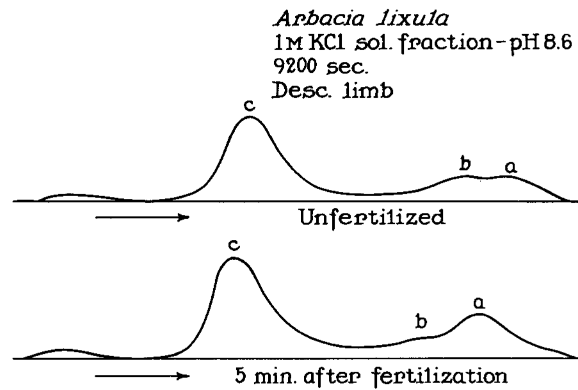

FIG. 2. Electrophoretic diagrams of the 1 M KCl soluble fraction (after removal of the water-soluble fraction) of unfertilized and fertilized eggs of *Arbacia lixula*.

fertilized and fertilized eggs. During the first 20 minutes following fertilization a decrease in the amount of N extractable with 1 M KCl occurs. In some cases, however, the N lost by the KCl fraction can be accounted for in the residue, whereas in other cases it is recovered in the water extract.

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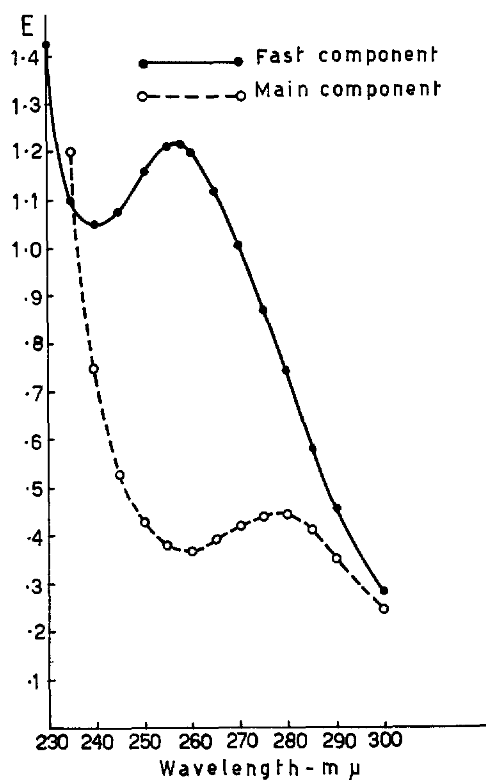

FIG. 3. Ultraviolet absorption spectrum of the group of the two fast moving components (*a* and *b*) and of the main component (*c*) of the KCl fraction.

TABLE II

Relative Concentrations of Protein Components of 1 M KCl Extracts of Eggs of Arbacia lixula

Concentrations in per cent of total proteins.

Preparations	Components			
	a $u = -13.6$	*b* $u = -11.5$	*c* $u = -5.8$	Ratio *a/b*
43,1 unfertilized	12.5	16.0	71.2	0.78
42,1 unfertilized	13.2	20.0	66.5	0.66
42,2 fertilized 5 min.	20.0	8.1	71.8	2.46
42,3 fertilized 10 min.	15.7	10.6	67.5	1.48

Electrophoresis in 0.1 N $NaHCO_3$, pH 8.6, ionic strength 0.1, potential gradient 4.6 volts/cm.

Preliminary experiments have shown that the extraction of the water-soluble protein fractions was complete within about 20 or 30 minutes at 0° C., and the yield did

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TABLE III

N Content (in Per Cent of the Total N of the Eggs) of the Water-Soluble and KCl-Soluble Fractions and of the Insoluble Residue of Unfertilized and Fertilized Eggs of Arbacia lixula

Preparation	Water N content	KCl N content	Residue N content
	mg. per cent	*mg. per cent*	*mg. per cent*
42,1 unfertilized	42.0	19.6	38.8
42,2 5 min. after fertilization	44.5	16.6	38.9
45,1 unfertilized	46.0	20.4	35.0
45,3 10 min. after fertilization	46.0	15.8	38.2
44,1 unfertilized	47.0	22.0	31.0
44,2 20 min. after fertilization	50.0	16.0	33.5
43,1 unfertilized	42.7	19.6	38.1
43,2 25 min. after fertilization	42.5	14.0	43.0

TABLE IV

Milligrams of N Extracted in Distilled Water from 100 Mg. of Unfertilized and Fertilized Eggs of Arabacia lixula at 0°C. and for Different Lengths of Time

Length of extraction	Unfertilized (batch 44,6) N extracted	Fertilized (batch 45,2) N extracted
min.	*mg.*	*mg.*
5	1.84	1.72
15	2.02	1.84
30	2.14	2.02
60	2.14	1.94

TABLE V

Milligrams of N Extracted with 1 M KCl from 100 Mg. of Unfertilized and Fertilized Eggs of Arbacia punctulata at 0°C. and for Different Lengths of Time

Length of extraction	Unfertilized N extracted	Fertilized N extracted
min.	*mg.*	*mg.*
10	6.64	6.50
45	6.16	6.77
60	6.96	6.58

not vary appreciably with longer extraction times (Table IV). Also the extraction of dry eggs with 1 M KCl goes to completion within about 30 minutes (Table V).

Mirsky showed (1936) that the protein fraction that becomes insoluble on fertilization can be induced to coagulate also when unfertilized eggs are frozen

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and thawed. We have found that on freezing and thawing the KCl extract (after removal of the salts by dialysis) a precipitate forms that can be separated by centrifugation. When examined electrophoretically, the soluble part shows a single component having the same mobility of the main component of the KCl fraction ($u = -5.9$) (Fig. 4). Sometimes a fast component at a low concentration can be observed also ($u = -13.1$). The coagulated fraction therefore is the complex of the components *a* and *b*. The analysis of the soluble fraction in the ultraviolet shows that the nucleic acid is now associated with it. Thus in the process of freezing and thawing the nucleic acid has been detached from the fraction undergoing coagulation. An estimate of

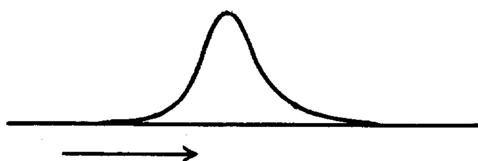

FIG. 4. Electrophoretic diagram of the soluble part of the KCl fraction after freezing and thawing. Electrophoresis in 0.1 N $NaHCO_3$, pH 8.6, ionic strength 0.1, potential gradient 4.6 volts/cm. after 4200 seconds.

TABLE VI

Coagulation of One Part of the KCl Fraction of Unfertilized Eggs of Arbacia lixula as Result of Extraction with Water at Room Temperature

Preparation	Water N extracted	KCl N extracted	Residue N extracted
	mg. per cent	*mg. per cent*	*mg. per cent*
Control; *i.e.*, extracted in the cold..............	46.1	20.4	35.0
Extracted for 30 min. at room temperature......	42.5	12.8	44.5

the N in the supernatant and in the precipitate obtained by freezing and thawing shows that the latter contains 33 per cent of the total N of the KCl fraction. This figure is in good agreement with the sum of the areas of components *a* and *b* of the electrophoretic diagram of the KCl fraction.

Mirsky also found (1937) that wetting of a frozen-dried preparation of muscle with water results in the coagulation of myosin. A decrease in the amount of material soluble in 1 M KCl is also observed when frozen-dried preparations of eggs of *Arbacia lixula* are extracted with distilled water at room temperature. A corresponding increase of the insoluble residue occurs. The data collected in Table VI show also a decrease in the water-soluble fraction. This has been observed in all the experiments to a more or less marked extent. Thus it seems that some other group of proteins besides those of the KCl fraction may also undergo coagulation under the conditions of the present experiments.

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Also, in these experiments the amount of the KCl fraction that may undergo coagulation amounts to about 36 per cent of the total KCl fraction. Therefore it appears that the ability to coagulate under different experimental conditions is confined to a certain group of proteins.

Some Experiments with the Eggs of Arbacia punctulata

In the course of the foregoing work, Dr. J. Runnström kindly informed us that experiments being carried out in his laboratory by S. Lindvall and A. Carsjö have shown that the sea water contained in the perivitelline space of the fertilized eggs, with its high salt content, must be held responsible for the coagulation of the sensitive fraction. Coagulation probably occurs during the process of freezing and drying or during the extraction. In order to check this possibility, eggs of *Arbacia punctulata* were collected during a stay at the

TABLE VII

N Content of the KCl-Soluble and Insoluble Fractions of Unfertilized and Fertilized Eggs of Arbacia punctulata

Preparation	KCl-soluble N content	KCl-insoluble N content
	mg. per cent	*mg. per cent*
112,1 unfertilized without jelly coat	80.5	19.5
112,2 30 min. after fertilization *with* fertilization membrane	80.8	19.2
112,3 30 min. after fertilization *without* fertilization membrane	81.0	19.0

Marine Biological Laboratory in Woods Hole. The eggs were freed of their jelly coats by treatment with slightly acid sea water and then treated with a dilute solution of crystalline trypsin in order to prevent the formation of the fertilization membrane by removing its precursor; *i.e.*, the vitelline membrane. After several washings with sea water, the eggs were inseminated. The eggs showed about 90 per cent fertilization. No fertilization membranes were formed. A control batch of eggs was collected in which only the jelly coat was removed. 30 minutes after fertilization the eggs were frozen at −80° C., after spreading them in a very thin layer, and were dried in a high vacuum as fast as possible.

The extraction of such eggs with 1 M KCl failed to show any change in the extractable proteins before and after fertilization (Table VII) not only in the eggs deprived of the fertilization membrane, but also in those in which this latter was present. Probably the way in which the material is frozen and dried is the critical factor in determining the results.

It is interesting to note here that the percentage of N extractable with 1 M

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KCl from the eggs of *Arbacia punctulata* from Woods Hole is much higher than from the eggs of *Arbacia lixula* from Naples.

The electrophoretic analysis of the total KCl extracts of these eggs did not show any appreciable difference between unfertilized and fertilized eggs. Centrifugation of such extracts at 25,000 × *g* results in the sedimentation of a fraction amounting to about 5 per cent of the total N of the unfertilized eggs and to about 4 per cent of the total N of the fertilized eggs. Referred to the total N of the unfertilized egg, this change—1.3 per cent—is very small indeed and could hardly be detected in an electrophoretic analysis. However, when working with the isolated fraction it becomes quite evident (Table VIII). We have found it constantly in three experiments. The difference was accounted for in the

TABLE VIII

N Content of the Fast Sedimenting Fraction from Unfertilized and Fertilized Eggs of Arbacia punctulata

Preparations	Fast sedimenting fraction N content	Supernatant N content	Residue N content
	mg. per cent	*mg. per cent*	*mg. per cent*
Unfertilized	5.26	75.7	19.2
30 min. after fertilization	4.14	77.0	18.7

supernatant of the centrifugation. Thus the part lost by the centrifugable fraction has become non-sedimentable in the centrifugal field used.

A preliminary analysis of the sedimented fraction carried out on a sample of unfertilized eggs, has given 13.5 per cent N, 2.4 per cent P. 75 per cent of the latter is accounted for as ribose P. No lipid P has been detected; however, 20 per cent by weight of this fraction is extractable in hot alcohol-ether.

CONCLUSIONS

The results presented in this paper give evidence of changes in solubility occurring in a protein fraction of the eggs of *Arbacia lixula* upon fertilization. The electrophoretic analysis indicates that it is only one part of one of the components of the KCl fraction that undergoes the change. However, under some experimental conditions (freezing and thawing of the KCl fraction or extraction of the whole eggs with water at room temperature) a larger portion of the KCl fraction, namely the whole group of components *a* and *b*, may be involved and undergoes coagulation. Therefore assuming that the results obtained on the extracts of frozen-dried fertilized eggs do reflect what actually occurs under natural conditions, we must also assume the existence of mechanisms controlling the extent of this change in the living eggs. The fact that in many cases one part or the whole of the sensitive fraction has been found to

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undergo an increase in solubility may suggest that the process of coagulation discovered by Mirsky is a two-step process. In the first step the sensitive fraction undergoes a change that makes it more soluble and then, when certain conditions are fulfilled, coagulation occurs. An alternative explanation could also be that the coagulated or coagulating fraction is attacked by the proteolytic enzyme that, as shown by Lundblad (1949, 1950), is activated on fertilization. This, however, seems to be less probable, as extraction was always carried out at 0° C. and in as short a time as possible.

However, further experiments are needed to decide whether the coagulation of the sensitive fraction is an actual occurrence under natural conditions. The results obtained with the eggs of *Arbacia punctulata* may cast some doubt on this assumption.

We wish to express our deepest thanks to Dr. A. E. Mirsky for the hospitality in his laboratory and for his continuous help and advice and for discussions in the course of this investigation. We should like also to thank Dr. L. G. Longsworth and Dr. G. E. Perlmann for the use of the electrophoretic equipment and for their help and advice.

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